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Phytic acid is the principle storage form of phosphorus comprising 1-5% by weight in cereals, legumes, oil seeds and nuts. In the forage, one-third of phosphorus is present as digestible inorganic P while two-third as organic P in the form of phytin®, which is a mixture of calcium-magnesium salts of inositolhexaphosphoric acid, known as phytic acid. Phytic acid and inositol intermediates have been implicated in starch digestibility and blood glucose response (Thompson, 1986), in the lowering of cholesterol and triglycerides (Jariwalla et al., 1990), in tumor formation (Shamsuddin et al., 1988; Ullah and Shamsuddin, 1990), in the treatment of Parkinson’s disease (Sabin, 1992), Alzheimer’s disease (Sabin, 1988; Sabin, 1989) and multiple sclerosis (Sabin, 1993). But this phytate phosphorus is largely unavailable to monogastric animals due to absence or insufficient amount of phytate degrading enzymes i.e., phytases in their gastrointestinal tract (Maenz and Iqbal et al., 1994; Classen, 1998; Boling et al., 2000) and since phytic acid can not be resorbed, feeds for pigs and poultry are commonly supplemented with inorganic phosphate in order to meet the phosphorus requirement (Reddy et al., 1982). Supplementation with inorganic phosphorus along with the phytate phosphorus excretion, however, imposes global ecological problems (eutrophication) when enters into rivers resulting in cyanobacterial blooms, hypoxia and death of marine animals and production of nitrous oxide, a potent green house gas (Erdman and Poneros, 1989; Mallin, 2000; Naqvi et al., 2000). Because of these problems, there is a considerable interest in phytate-degrading enzymes i.e., phytases which hydrolyses the phosphate moieties from phytate, thereby resulting in loss of ability of phytic acid to chelate metal ions. Also, the supplementation of phytase in fodder improves the phosphorus bioavailability besides reducing phosphorus excretion in the areas of intensive livestock (Common, 1989; Ward, 1993; Yano et al., 1999). Thus, for both environmental and economic reasons, phytases and phytase producing microbes are attracting significant industrial interest.

Phytases (myo-inositol hexakisphosphate phosphohydrolase; EC 3.1.3.8 and EC 3.1.3.26) catalyze the hydrolysis of phytic acid in a stepwise manner to lower inositol phosphates, myo-inositol and inorganic phosphate, all utilizing a phosphohistidine intermediate in their phosphoryl transfer reaction (Mitchell et al., 1997). Thus, phytases become potential candidate for the production of special isomers of different lower phosphate esters of
myo-inositol, some of which are considered to be pharmacoactive and important intracellular secondary messengers (Greiner and Konietzny, 1996). The research on phytase spans 87 years from its discovery by Suzuki et al., (1907) until its commercialization in Europe in 1994 by Gist-Brocades, which emphasized not only on practical use and delivery of enzyme but also the ability to produce the enzyme economically. At the close of twentieth century, annual sales of phytase as animal feed additive were estimated to be $500 million, rising further (Abelson, 1999). Thus the growth of the market for phosphate animal feed additive fostered a critical step in the commercial development of phytases, which are widely distributed in nature.

1. CLASSIFICATION OF PHYTASE
The International Union of Biochemistry and Molecular Biology (IUBMB) in consultation with the IUPAC-IUB, Joint Commission on Biochemical Nomenclature (JCBN) listed two types of phytases:

EC 3.1.3.8
Recommended name: a 3-phytase
Systematic name: myo- inositolhexakisphosphate-3-phosphohydrolase
It hydrolyzes the ester bond at the 3rd position of myo- inositolhexakisphosphate to D-myoinositol-1,2,4,5,6-pentakisphosphate and orthophosphate.

EC 3.1.3.26
Recommended name: a 6-phytase
Systematic name: myo- inositolhexakisphosphate-6-phosphohydrolase
It hydrolyzes the ester bond at the 6th position of myo- inositolhexakisphosphate to D-myoinositol-1,2,3,4,5-pentakisphosphate and orthophosphate. Subsequent ester bonds in the substrate were hydrolyzed at different rates.

1.1 Phytases that are histidine acid phosphatases (HAPs)
Several types of acid phosphatases have been reported in the biological systems. These include purple acid phosphatases, with Fe-Fe or Fe-Zn in their active site (Klabunde et al., 1996), the low molecular weight acid phosphatases and the high molecular weight acid phosphatases (Vincent et al., 1992).
1.1.1 Phy A

When *Aspergillus niger* NRRL 3135 was grown on cornstarch based media (Shieh and Ware, 1968) it produced highest amount of extracellular phytase. When this organism was grown under phosphate limiting conditions it produces three extracellular acid phosphatases (Ullah and Cummins, 1988). One is characterized as phytase (phy A) with two pH optima, 2.5 and 5.0. The phy A produced by *Aspergillus niger* NRRL 3135 had conserved active site motif, RHGXRXP, which is unique to high molecular weight acid phosphatase class of enzyme and hydrolyzes the phosphomonoesters in a two-step mechanism (Ullah *et al.*, 1991; Van Etten *et al.*, 1991). The phy A from *A. niger* NRRL 3135 is a monomeric protein of 48.5 kDa for unglycosylated enzyme (Ullah and Dischinger, 1993). However, the native enzyme is heavily glycosylated with a molecular weight of 85 kDa (Ullah, 1988). The enzyme showed inherent thermostability possessing optimum temperature of 58°C (Ullah and Gibson, 1987) with 10 cysteine residues involved in forming 5 disulfide bridges (Ullah and Mullaney, 1996; Kostrewa *et al.*, 1997). Van Etten *et al.*, (1991) first recognized another hallmark of phy A protein i.e., C-terminal HD motif (His 361 and Asp 362). The HD motif is well conserved in wide variety of phytase sequences (Ehrlich *et al.*, 1993; Mitchell *et al.*, 1997; Pasamontes *et al.*, 1997). The complete amino acid sequence of phytase A was shown in Figure 1. The enzyme was found to remain stable for months in crude culture filtrate. The role of glycosylation in the functional expression of *A. niger* phy A in *Pichia pastoris* was investigated by Han and Lei (1999) and found it to be vital for enzyme’s thermostability. Wyss *et al.*, (1999b) studied the effect of glycosylation pattern on specific activity, thermostability and refolding properties of several fungal phyAs. The expression of gene encoding fungal phy A in *E. coli*, where glycosylation would not be possible, resulted in inactive protein which did not fold properly, thus, suggesting that glycosylation may be involved in assisting enzyme folding. This may explain why the fungal phytases has not been successfully cloned and expressed in bacteria. In 1996, a process for deglycosylation of proteins for crystallization using recombinant glycosidase fusion protein was developed (Grueninger-Leitch *et al.*, 1996) that enabled Kostrewa *et al.*, (1997) to first crystallize *A. niger* NRRL 3135 phytase (phy A) and determine its crystal structure by X-ray crystallography.
**Figure 1:** The amino acid sequence of *Aspergillus niger* NRRL 3135 phytase (NCBI Accession No. JN0656)

(The conserved residues having side chains protruding into the reaction cavity have an asterisk (*) above them (Pasamontes et al., 1997b) and the # above the 10 Asn residues denotes glycosylation. The N-terminal (N) RHGXRXP and C-terminal HD motifs in histidine acid phosphatase are coloured blue. The two acidic and four basic amino acids of substrate specificity site are coloured pink.)
1.1.2 Phy B

The second enzyme produced by *A. niger* NRRL 3135 with phytase activity, which is also HAP, is phytase B (phy B) and was initially referred as pH 2.5 optimum acid phosphatase. Sequence alignment studies of phy A and phy B indicated a very similar active site with the sequence motif RHGXRXP. The enzyme lack phytate degrading activity at pH 5.0 while at pH 2.5, it efficiently hydrolyzes phytate with a turnover number of 628 second⁻¹ as compared to 348 second⁻¹ for phy A (Ullah and Phillippy, 1994). This was attributed to differences in the charge distribution at the substrate specificity sites of phy A and phy B (Kostrewa *et al.*, 1999). In phy B, there are only two acidic amino acids, Asp 75 and Glu 272 (Figure 2) while in phy A substrate specificity site, there are two acidic and four basic amino acids viz., Glu 228, Asp 262, Lys 91, Lys 94, Lys 300 and Lys 301 (Figure 1). As the active site of phy B was more acidic than phy A, the acidic amino acids were uncharged at pH 2.5 and can accommodate the negatively charged phytate as substrate while in phy A, four basic amino acids (Lys 91, Lys 94, Lys 300 and Lys 301) were all positively charged that would attract the negatively charged phytate. When the pH was raised to 5.0, the acidic amino acids become negatively charged while basic amino acids remain positively charged. On the contrary, substrate-binding site of phy B would repel negatively charged phytate molecule while the site in phy A would still attract the phosphate groups of phytate. Also, phy B exhibit broader substrate specificity than phy A (Ullah and Cummins, 1988; Wyss *et al.*, 1999a). This was attributed to neutral electrostatic field of phy B site where wide variety of phosphomonoesters can be utilized while the highly positive electrostatic field of phy A’s substrate binding site would accommodate negatively charged phytate. Consequently, other less charged substrates bind less effectively at that site (Kostrewa *et al.*, 1999). The crystal structure of *A. niger* T213 showed phy B to be a tetramer formed of two dimers that allows each active site ready access to the substrates. Besides this, phy B from *Aspergillus niger* T213 is more thermostable than recombinant phy A from either *Aspergillus niger* T213 or *Aspergillus fumigatus* ATCC 13070 (Wyss *et al.*, 1998).

1.1.3 E. coli HAP

Dassa and Boquet (1985) first identified the periplasmic acid phosphatase encoded by *E. coli* appA gene, with 2.5 pH optimum. Subsequently, it was denoted as phytase P2,
Figure 2: The amino acid sequence from the crystal structure study of the *Aspergillus niger* T213 phyB phytase gene (Kostrewa et al., 1999)

(The N-terminal (N) RHGXRXRXP and C-terminal HD motifs in histidine acid phosphatase are coloured blue. The acidic amino acids of substrate specificity site are pink. The dark red residues indicate the start of N-terminus following the 19-residue signal sequence).
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because of its high phytase activity and later, established as 6-phytase (Greiner et al., 1993). The complete nucleotide sequence of *E. coli* appA gene was determined to utilize it for cloning the phytase gene (Dassa et al., 1990). Rodriguez et al., (1999) cloned the phytase gene from *E. coli* and found that the nucleotide sequence, designated as appA2, was 95% homologous to appA. When cloned and expressed in *Pichia pastoris*, both r-appA and r-appA2 proteins were markedly different in their pH profile and other catalytic properties, despite having identical sequences in the regions of the N-terminal motif (RHGVRAP, 38-44) and the C-terminal motif (HD, 325-326). The role of C-terminal HD motif in the catalysis of HAPs by site directed mutagenesis was demonstrated by Ostanin and Van Etten (1993).

1.1.4 Yeast HAP

Phytases having HAPs motifs were also reported in several yeast species. Three yeast acid phosphatases were reported to have both RHGXRXP and HD motif in their amino-acid sequence, which is the characteristic feature of HAPs. The gene encoding phytase (pho3) was first reported in *Saccharomyces cerevisiae* (EC 3.1.3.2) by Bajwa et al., (1984) translating to 467-residue active protein. Later, phytase genes viz., *Schizosaccharomyces pombe* pho1 and *Schizosaccharomyces pombe* pho 4 resulting in active 453 and 463-residue protein, respectively were identified (Elliot et al., 1986; Yang and Schweingruber, 1990). The *S. cerevisiae* pho3 gene was, later, cloned and transformed into *Aspergillus oryzae* expression system, which resulted in four-six fold increase in phytase activity.

1.1.5 Plant HAP

Maugenest et al., (1997) cloned and sequenced phytase cDNA, phy S11, in maize and utilized it to screen the maize genomic library. Two phytase genes, *PHYT I* and *PHYT II* were identified and found to express in monocotyledons. But the transcribed sequences of both genes share little homology with *Aspergillus niger* phytase except around HAP consensus motif, RHGXRXP (Maugenest et al., 1999). A putative *Arabidopsis thaliana* phytase, with both RHGXRXP and HD active sequence was identified. The enzyme showed homology with *A. niger* phy A exhibiting 10 cysteine involved in the formation of 5 disulfide bonds, necessary for proper protein folding (Mullaney and Ullah, 1998b).
1.2 Phytases with undefined active site

Several phytases were reported in literature that did not possess RHGXRXP and HD motif, found in the active site of HAPs and thus, have different requirements for their catalytic action.

1.2.1 Phy C

Powar and Jagannathan (1982) reported phytase from Bacillus subtilis having requirement of Ca$^{2+}$ for catalysis and showed optimum activity at pH 7.5. Later, a thermostable phytase from Bacillus amyloliquefaciens was purified and characterized by Kim et al., (1998a) after which its gene was cloned and over expressed in E. coli (Kim et al., 1998b). Kerovuo et al., (1998) purified and characterized phytase from Bacillus subtilis VTTE-68013 and named it as phy C. Analysis of the protein sequence indicated that it did not share any active site motif found in HAPs and required Ca$^{2+}$ for catalysis similar to B. amyloliquefaciens though both varied in optimum temperatures: 55°C for B. subtilis VTTE-68013 and 70°C for B. amyloliquefaciens.

1.2.2 Klebsiella phytase

Greiner et al., (1997) reported a monomeric 40 kDa, 3-phytase from Klebsiella terrigena, which like phy A had optimum temperature of 58°C and is not metalloenzyme. Tambe et al., (1994) found two inducible molecular forms of phytase from Klebsiella aerogenes having molecular weight of 700 kDa with optimum pH of 4.5 and 10-13 kDa with optimum pH of 5.2 for phytase activity.

1.2.3 Yeast phytase

Segueilha et al., (1992) reported secretory phytase from Schwanniomyces castellii having molecular weight of 490 kDa. The enzyme was found to be tetrameric with one large subunit of 125 kDa and three identical subunits of 70 kDa. Sano et al., (1999) reported the secretory phytase from Arxula adeninivorans having optimum temperature and pH of 75°C and 4.5, respectively for its activity. The level of secreted enzyme far exceeded that of previously reported yeast phytases but the enzyme was not heat resistant in the absence of substrate phytate. Lambrechts et al., (1993) optimized the conditions for the production of Schwanniomyces castellii CBS 2863 phytase and found that the level of production was significantly influenced by phytic acid or phosphate content. The enzyme had the optimum temperature of 77°C for catalytic activity.
1.2.4 Plant phytase

The phytase from soybean was purified and characterized by Morgan et al., (1998). The enzyme did not show sequence homology with any of the known histidine acid phosphatase and was reported to be homologous to the N-terminus of purple acid phosphatase of Arabidopsis thalliana. Another phytase from Avena sativa was isolated having molecular weight of 67 kDa with pH and temperature optima of 5.0 and 35°C, respectively (Greiner and Alminger, 1999).

2. SOURCES OF PHYTASE

Phytases can be derived from a number of sources including plants, animals and microorganisms. Recent research has shown that microbial sources are more promising for the production of phytases on a commercial scale. Table 1 cites several reports on the production of phytases from microbial sources.

2.1 Bacteria

Several bacterial strains have been examined for the production of phytase viz., Bacillus subtilis, B. amyloliquefaciens, E. coli, Pseudomonas sp., Klebsiella sp., Lactobacillus amyllovorans etc. Yoon et al., (1996) reported phytase production from Enterobacter sp.4 in minimal salt medium at 37°C, pH 5.5. The enzyme showed optimum activity at 50°C, pH 7.0-7.5 and was inhibited by Zn^{2+}, Ba^{2+}, Cu^{2+}, Al^{3+} and EDTA. A genetically modified B. subtilis produced 100-fold higher extracellular phytase than the wild type B. amyloliquefaciens DS11 and was very specific for phytate, having no or little activity towards other phosphates, showing optimum activity at 70°C, pH 7.0 with isoelectric point of 5.3 and required Ca^{2+} for thermal stability (Kim et al., 1998b; Kim et al., 1999). Shimizu (1992) reported the production of extracellular phytase exhibiting optimum activity at 60°C, pH 6.0-6.5 with isoelectric point (pI) of 6.25 and Ca^{2+} requirement for the production and activity, from Bacillus subtilis (natto) N-77 and found it to be fairly specific for phytate. Kerovuo et al., (1998) isolated, purified, characterized and cloned extracellular phytase from Bacillus subtilis VTT E-68013. The enzyme had maximal activity at 55°C, pH 7.0, required Ca^{2+} for activity and stability and showed no homology with the active site sequence of HAPs, thus, designated as phy C having phytase activity. Greiner et al., (1993) purified two periplasmic phytases, P1 and P2, from E. coli showing
TABLE 1: SOURCES OF MICROBIAL PHYTASE

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Expressed in</th>
<th>Production Technique</th>
<th>References</th>
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<tr>
<td><strong>Bacteria</strong></td>
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<tr>
<td>B. amyloliquefaciens</td>
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<td>Kim et al., (1999b)</td>
</tr>
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<td>Yoon et al., (1996)</td>
</tr>
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<td>E. coli</td>
<td>-</td>
<td>-</td>
<td>Greiner and Jany (1991); Jia et al., (1998); Wyss et al., (1999); Lim et al., (2000); Golovan et al., (2000)</td>
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<td>K. aerogenes</td>
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<td>K. terrigena</td>
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<td>-</td>
<td>Greiner et al., (1997)</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>Yanke et al., (1998)</td>
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<tr>
<td>Pseudomonas sp.</td>
<td>-</td>
<td>-</td>
<td>Richardson and Hadobas (1997)</td>
</tr>
<tr>
<td>Treponema sp.*</td>
<td>-</td>
<td>-</td>
<td>Yanke et al., (1998); Cheng et al., (1999)</td>
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<tr>
<td><strong>Yeasts</strong></td>
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<tr>
<td>Arxula adeninivorans</td>
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<td>Foods</td>
<td>Fermentation Process</td>
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<td><em>A. carbonarius</em></td>
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<td>Ebune <em>et al.</em>, (1995)</td>
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<td>Han <em>et al.</em>, (1999)</td>
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<td>Han and Lei (1999)</td>
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<td><em>A. niger</em></td>
<td>Pichia pastoris</td>
<td>-</td>
<td>Shimizu (1993)</td>
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<td><em>A. oryzae</em></td>
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<td><em>A. terreus</em></td>
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<tr>
<td><em>Thermomyces lanuginose</em></td>
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*Anaerobic bacteria; SmF-Submerged fermentation; SSF-Solid state fermentation.*
maximum activity at 55°C, pH 4.5. The phytase activity was investigated by Yanke et al., (1998) in several strains of anaerobic ruminal bacteria viz., *Selenomonas ruminantium, Megasphaera elsdenii, Preotella ruminicola, Mitsukella multiacidus* and *Treponema* sp. In general, phytases produced by bacteria had pH optimum in neutral to alkaline range with low yield that precludes their use as feed additives.

### 2.2 Fungi

Over 200 fungal isolates belonging to genera *Aspergillus, Mucor, Pencillium* and *Rhizopus* have been tested for phytase production (Howson and Davis, 1983; Gargova et al., 1997). In this survey of phytate-degrading microorganisms (from plants, animals and microorganisms), *Aspergillus ficuum* NRRL 3135 produced highest amount of enzyme on cornstarch based media (Shieh and Ware, 1968). The organism produced three extracellular acid phosphatases when grown under phosphate limiting conditions (Ullah and Gibson, 1987; Ullah and Cummins, 1988). One is characterized as phytase (phy A) with two pH optima, 5.0 and 2.5 while the second enzyme (phy B) was referred to as pH 2.5 optimum acid phosphatase. Sequence alignment studies of phy A and phy B indicated a very similar active site with the sequence motif RHGXRXP. The third enzyme, designated as pH 6.0-optimum acid phosphatase, showed no homology to active site of phy A and phy B, and lacks any detectable phytase activity. The phytase produced by *A. niger* NRRL 3135 has been well characterized (Ullah and Gibson, 1987; Ullah, 1988) and phy A, has been cloned from *A. niger* (Mullaney et al., 1991). In addition, the cloning and expression of the phy A gene have been reported in *A. awamori* (Piddington et al., 1993), *A. ficuum* (van Hartingsveldt et al., 1993) and in *A. terreus* (Mitchell et al., 1997). Later, Han et al., (1999) successfully cloned and expressed *Aspergillus niger* phy A gene in *S. cerevisiae* as an active, extracellular, heavily glycosylated phytase having two pH optima (2.0-2.5 and 5.0-5.5) with temperature optimum at 55-60°C. Berka et al., (1998) cloned phy A gene encoding extracellular phytase from the thermophilic fungus *Thermomyces lanuginosus* and heterologously expressed in *Fusarium venenatum*. Wyss et al., (1998) studied the properties of three HAPs from *A. fumigatus, A. niger* and *A. niger* pH 2.5 optimum acid phosphatase. Later, they studied the biophysical properties of six different fungi viz., *A. niger, A. terreus, A. fumigatus, Emericella nidulans,*
Myceliophthora thermophila and Talaromyces thermophilus (Wyss et al., 1999a) which were either overexpressed in filamentous fungi or yeasts.

2.3 Yeast
Relatively few studies have been reported on the production of phytase from yeast sp. viz., Schwanniomyces castellii CBS 2863, Arxula adeninivorans, Hansenula polymorpha, Rhodotorula gracilis. Sano et al., (1999) screened 1200 yeast strains from CBS collection for their capability to grow on phytic acid, as sole source of carbon and phosphate. The strains belonging to Arxula adeninivorans were found to be best assimilator and were capable of secreting high levels of phytase into the culture medium at 44°C when glucose was replaced by galactose. The secreted enzyme was found to be optimally active at 75°C and in the pH range of 4.5-5.0. Nakamura et al., (2000) reported phytase production from several strains viz., Pichia, Candida, Kluyveromyces, Torulaspora, Schwanniomyces sp. and found that yeast enzymes had an optimal activity at pH 4.0-5.0 and generally, very high optimal temperature ranging from 60-80°C. S. castellii CBS 2863 secreted high level of phytase and was found to be dependent on the medium composition i.e., cations, phosphate and phytic acid (Lambrechts et al.,1992; Lambrechts et al.,1993). Mayer et al., (1999) developed the process for the low cost production of phytase, using wild type phytase genes of Aspergillus fumigatus (Pasamontes et al., 1997) and A. terreus (Mitchell et al., 1997), transformed in Hansenula polymorpha. Both the fermentation and downstream processing were tested in pilot scale upto 2000 L.

2.4 Plants and animal
Phytases have been reported in rice (Oryzae sativa), wheat (Triticum aestivum), maize (Zea mays), soybean, dwarf beans, mung beans, rye (Secale cereale), and other legumes or oil seeds (Eskin et al., 1983; Gibson and Ullah, 1990; Laboure et al., 1993). In soybean, an approximately ten-fold increase in phytase activity was observed, with a maximum level of activity at 8-10 days of post-germination (Gibson and Ullah, 1988). Nayini and Markakis (1986) also observed similar increase in phytase activity with the concomitant decrease in phytic acid content during germination but whether it was due to the activation of pre-existing enzyme or de novo synthesis of protein, is still uncertain.

As compared to phytases from microbial sources, very little is known about animal phytases though reports on creating transgenic animals for efficiently metabolizing phosphates from feeds are there. Golovan *et al.*, (2001) produced transgenic mice that expressed *E.coli* appA phytase gene in salivary gland and secreted biologically active 55 kDa, glycosylated protein in saliva. The transgenes were regulated either by the inducible rat R15 proline-rich protein (PRP) promoter (Tu *et al.*, 1993) or by the constitutive mouse parotid secretory protein (PSP) promoter (Laursen and Hjorth, 1997). Expression of salivary phytase leads to significant reduction of fecal phosphorus, suggesting it to be promising approach to relieve requirements for dietary phosphate supplementation and phosphorus pollution from animal agriculture.

### 3. PRODUCTION OF MICROBIAL PHYTASE

#### 3.1 Production techniques

Techniques using solid-state fermentation (SSF) and submerged fermentation (SmF) have been employed for phytase production. But the culture conditions, type of strain, nature of substrate and availability of nutrients should be taken into consideration for selecting a particular production technique, as they are the critical factors affecting the yield. The
production of phytase from *A. ficuum* NRRL 3135 has been achieved by three different cultivation methods, namely, solid state (Ebune *et al.*, 1995), semi-solid (Han *et al.*, 1987) and submerged fermentations (Howson and Davis, 1983; Ullah and Gibson, 1987). In the submerged batch culture, the production of phytase was inhibited by high concentration of glucose and low level of aeration (Shieh and Ware, 1968). This can be overcome by utilizing a fed-batch operation in due course. SSF of canola meal using *A. niger* NRRL 3135 was reported to reduce the phytic acid content with complete reduction in 2 days of fermentation (Nair and Duvnjak, 1990; Nair and Duvnjak, 1991). Ebune *et al.*, (1995) also studied the phytase production by *A. ficuum* using canola meal and found that the age of inoculum had profound effect on enzyme synthesis. Krishna and Nokes (2001) studied the effect of culture conditions, particularly inoculum age, media composition (wheat bran and full-fat soybean flour) and duration of SSF on the phytase production by *Aspergillus niger* using response surface methodology. Phytase production using SmF from *Bacillus* sp. DS11 in the medium consisting of wheat bran and casein hydrolysate as carbon and nitrogen source produced thermostable, extracellular phytase at 37°C (Kim *et al.*, 1998). The phytase gene was then cloned in *Bacillus subtilis* which resulted in 100 times higher phytase yield in modified Luria broth medium that contained, in addition to LB medium, 4% glucose and metal salts (Kim *et al.*, 1999a). Kim *et al.*, (1999b) studied phytase production from *Aspergillus* sp.5990, higher optimum temperature for catalytic activity than the commercial Natuphos from *A. ficuum* NRRL 3135, using SmF at 37°C, pH 7.0 where five-fold higher activity in liquid culture was obtained. Papagianni *et al.*, (2000) investigated qualitative relationship between medium composition, morphology and phytase production by *Aspergillus niger*.

### 3.2 Effect of phosphorus concentration

High phosphate conditions are known to repress the synthesis of acid phosphatases and phytases, while limiting phosphate conditions result in their expression. In a survey of phytase producing microorganisms (Sheih *et al.*, 1969; Sheih and Ware, 1968), *Aspergillus ficuum* produced highest amount of phytase (113 nkat/ml in shake flask in 5 days) when the inorganic phosphorus content was controlled in the range of 0.0001-0.005%, optimum being 0.4 mg/100 ml phosphorus and 8% cornstarch. Han and
Review of Literature

Gallagher (1987) also confirmed that high phosphorus concentration inhibited phytase synthesis by *A. niger* NRRL 3135. They noted that 1-5 mg P/100 ml was needed for maximum phytase production while 8 mg P/100 ml medium was required for maximum cell growth. Utt (1987) tested eight different sources of commercial cornstarch on phytase yield where difference of 5.2 fold was observed depending on the cornstarch source. Gibson (1987) also confirmed the effect of phosphorus level in the medium and compared the production from several sources, speculating that the phosphoester linkage in some starch sources may be more resistant to cleavage than others, resulting in low but steady supply of phosphorus. Han *et al.* (1987) obtained similar trend for phytase production by *A. ficuum* on semisolid substrate using soybean meal where 10 mg P/100 g substrate in the growth medium resulted in high phytase activity (82.5 U/g substrate) as compared to a control without added phosphate (8.0 U/g substrate) while higher phosphate levels inhibited phytase production. Chelius and Wodzinski (1994) during the strain improvement study of *A. niger* NRRL 3135 by UV radiation, reported that phy A production in the mutant strain was repressed 60% when the P concentration was 0.006 % (w/v) whereas phytase production in wild type strain was not influenced significantly by the addition of increasing concentrations of phosphorus from 0.006-0.015% (w/v). Gargova *et al.*, (1997) reported extracellular phytase having dual optima (pH 5.0 and 2.5) from *Aspergillus* sp. 307 where maximum secretion took place at 20mg/dm³ phosphorus above, which decline in production, was observed. Kim *et al.*, (1999b) showed that extracellular phytase production by *Aspergillus* sp.5990 was maximally obtained (18 μmole/minute) at 50mg/L phosphate concentration or below while production was greatly reduced at phosphate concentration greater than 100 mg/L.

3.3 Effect of medium ingredients and inoculum

Al-Asheh and Duvnjak (1994) studied the effect of surfactants such as Tween-80, Triton-X-100, sodium olate on the phytase production and reduction of phytic acid content in canola meal (37-40 % protein and 4-6 % phytic acid) by *Aspergillus carbonarius* in SSF. The phytase production increased in the presence of 1% sodium olate and Tween-80 suggesting that these surfactants alter the cell permeability, resulting in the higher release of enzyme. Han and Gallagher (1987) tested the same surfactants for the phytase
production by *A. ficuum* in liquid medium and reported that besides, dispersed growth of mycelium, an increase in phytase level of 1.3, 1.7 and 4.8 times resulted, when 0.5% of Triton-X-100, Tween-80 and Na-oleate, respectively were added to the medium. Similarly, Mandviwala and Khire (2000) reported 30% increase in phytase activity of *A. niger* NCIM 563 when 0.5% Triton-X-100 was added to the production medium. Also, the inoculum size and inoculum quality are important factors for the enzyme production. If either the inoculum size is too small or if relatively, low viscosity medium devoid of cornstarch is used, the organism forms mycelial pellets resulting in decline in phytase production (Wodzinski and Ullah, 1996). Krishna and Nokes (2001) demonstrated the impact of inoculum culturing conditions on phytase production and overall performance of the fermentation process during SSF by *Aspergillus niger* using response surface methodology. They reported that phytase production was strongly growth associated in SSF where younger fungal inoculum, being in active growth phase, contributes to the active utilization of the substrate and further product formation.

### 3.4 Mutation studies

Comparatively few reports are published regarding improvement of phytase production via mutagenesis. Chelius and Wodzinski (1994) during the strain improvement study of *A. niger* NRRL 3135 by UV radiation, isolated a phytase catalytic mutant producing 3.3-fold higher phytase (phy A) than the wild type strain. The production of mutant phy A was repressed 60% by the inorganic phosphate (0.006%, w/v) however, their approach was limited by lack of specificity and sensitivity to discriminate phytase and acid-phosphatase activity during primary screening process. Thus, a selection method that would differentiate phytase activity from acid-hydrolysis, would improve the efficiency of developing phytase-producing strain.

### 4. BIOCHEMICAL CHARACTERIZATION OF PHYTASE

The physicochemical characteristics and catalytic properties of phytases from various sources are listed in Table 2. Phytase is an ester-hydrolyzing enzyme with an estimated molecular weight of 35-700 kDa depending upon the source of origin and are usually active within pH range of 4.5-6.0.
# TABLE 2: PHYSICOCHEMICAL AND KINETIC PROPERTIES OF PHYTASE

<table>
<thead>
<tr>
<th>Sources</th>
<th>Molecular weight (kDa)</th>
<th>Isoelectric point (pI)</th>
<th>Optimum pH</th>
<th>Temp (°C)</th>
<th>Substrate Selectivity</th>
<th>K_{cat} (s^{-1})</th>
<th>K_{m} (mM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Bacillus sp. DS11</td>
<td>44</td>
<td>5.3</td>
<td>7.0</td>
<td>70</td>
<td>Specific</td>
<td>-</td>
<td>0.55</td>
<td>Kim <em>et al.</em>, (1998)</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>36-38</td>
<td>6.25</td>
<td>6.0-6.5</td>
<td>60</td>
<td>Specific</td>
<td>5.5</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Enterobacter sp.</td>
<td>42</td>
<td>6.3-6.5</td>
<td>4.5</td>
<td>60</td>
<td>Specific</td>
<td>6209</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
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<tr>
<td>K. aerogenes</td>
<td>10-13, 700</td>
<td>10.5, 3.7</td>
<td>5.2, 4.5</td>
<td>60-70</td>
<td>Specific</td>
<td>-</td>
<td>0.114, 0.062</td>
<td></td>
</tr>
<tr>
<td>K. terrigena</td>
<td>40</td>
<td></td>
<td>5.0</td>
<td>58</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>Mitsuokella multiacidus</td>
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<tr>
<td>Prevotella ruminicola</td>
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<td>Selenomonas ruminantium</td>
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<td>Yeasts</td>
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<tr>
<td><em>Arxula adeninivorans</em></td>
<td>-</td>
<td>-</td>
<td>4.5</td>
<td>75</td>
<td>Specific</td>
<td>-</td>
<td>0.23</td>
<td>Sano et al., (1999)</td>
</tr>
<tr>
<td><em>Schwanniomyces castellii</em></td>
<td>490</td>
<td>-</td>
<td>4.4</td>
<td>77</td>
<td>Specific</td>
<td>-</td>
<td>0.038</td>
<td>Segueilha et al., (1993)</td>
</tr>
<tr>
<td><em>S. occidentalis</em></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Nakamura et al., (1999)</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>120</td>
<td>-</td>
<td>2.0-2.5, 55-60</td>
<td>5.0-5.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Han et al., (1999)</td>
</tr>
<tr>
<td><em>Pichia pastoris</em></td>
<td>95</td>
<td>-</td>
<td>2.5-5.5</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Han and Lei (1999)</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
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</tr>
<tr>
<td><em>A. ficuum</em> (phy A)</td>
<td>85</td>
<td>4.5</td>
<td>2.5-5.0</td>
<td>58</td>
<td>Specific</td>
<td>348</td>
<td>0.027</td>
<td>Ullah and Gibson (1987)</td>
</tr>
<tr>
<td><em>A. ficuum</em> (phy B)</td>
<td>68</td>
<td>4.0</td>
<td>2.5</td>
<td>63</td>
<td>Broad</td>
<td>628</td>
<td>0.103</td>
<td>Ullah and Cummins (1987)</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>100</td>
<td>-</td>
<td>5.0</td>
<td>55</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Dvorakova et al., (1997)</td>
</tr>
<tr>
<td><em>A. niger SK-57</em></td>
<td>60</td>
<td>-</td>
<td>5.5-2.5</td>
<td>50</td>
<td>Specific</td>
<td>-</td>
<td>0.0187</td>
<td>Nagashima et al., (1999).</td>
</tr>
<tr>
<td><em>A. oryzae</em></td>
<td>120</td>
<td>4.15</td>
<td>5.5</td>
<td>50</td>
<td>Broad</td>
<td>-</td>
<td>0.33</td>
<td>Shimizu (1993)</td>
</tr>
<tr>
<td>Plants</td>
<td></td>
<td></td>
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<tr>
<td>Canola seed</td>
<td>-</td>
<td>-</td>
<td>5.2</td>
<td>50</td>
<td>Broad</td>
<td>-</td>
<td>-</td>
<td>Krishna and Nokes (2001)</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>76</td>
<td>-</td>
<td>4.8</td>
<td>55</td>
<td>Broad</td>
<td>-</td>
<td>0.117</td>
<td>Laboure et al., (1993)</td>
</tr>
<tr>
<td><em>Soybean</em></td>
<td>60</td>
<td>5.5</td>
<td>4.5-4.8</td>
<td>55</td>
<td>Broad</td>
<td>-</td>
<td>0.048</td>
<td>Gibson and Ullah (1988)</td>
</tr>
<tr>
<td><em>Spelt</em></td>
<td>68</td>
<td>-</td>
<td>6.0-7.0</td>
<td>45</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Konietzny et al., (1995)</td>
</tr>
<tr>
<td><em>Wheat</em></td>
<td>-</td>
<td>-</td>
<td>5.1</td>
<td>55</td>
<td>Broad</td>
<td>-</td>
<td>0.55</td>
<td>Khare et al., (1994)</td>
</tr>
</tbody>
</table>

**Review of Literature**
4.1 Purification and characterization

Aspergillus niger NRRL 3135 phytases viz., phy A, phy B and pH 6.0-optimum acid phosphatase are secretory proteins and their relative amounts are considerably higher when grown under phosphate starvation condition in starch medium. The purification profile of these proteins (Ullah and Gibson, 1987; Ullah and Cummins, 1987b; Ullah and Cummins, 1988) indicated that approximately 50% of the total secreted proteins were phy A, phy B and the pH 6.0-optimum acid phosphatases. The secreted glycoproteins were stable for many months at 5°C and lack any intrinsic protease activity, thus allowing the purification at room temperature using ion-exchange chromatography and chromatofocusing. These proteins were found to be microheterogenous due to differential glycosylation with estimated molecular weight of monomer to be 85, 65 and 85 kDa for phy A, phy B and pH 6.0-optimum acid-phosphatase, respectively by SDS-PAGE. The $K_{cat}/K_m$ ratio of fungal phytases for myo-inositol hexa-, penta-, tetra- and tri-phosphate indicated myo-inositolhexaphosphate as preferred substrate for both phy A and phy B, thus, categorizing them to be phytase (Ullah and Phillippy, 1994).

An extracellular phytase from Bacillus subtilis (natto) N-77 was purified 322-fold to homogeneity by ultra-filtration and a combination of Sephadex G-100 and DEAE-Sepharose CL-6B column chromatography. The molecular weight of purified protein was found to be 36 kDa on gel-filtration and 38 kDa on SDS-PAGE, suggesting the native enzyme to be a monomer. The enzyme had $K_m$ of 0.5 mM, 6.25 $\mu$M with pH and temperature optimum at 6.0-6.5, 60°C, respectively (Shimizu, 1992). Kerovuo et al., (1998) purified phytase (phyC) from Bacillus subtilis VTT E-68013 showing optimum temperature and pH at 55°C, 7.0 respectively. The purified enzyme was found to be metal-ion dependent as it required calcium for activity and stability. Kerovuo et al., (2000) also studied the metal-ion requirement of Bacillus subtilis phytase that showed complete inactivation of enzyme upon removal of metal ions by EDTA but the phytase activity was partially restored when incubated with calcium. The inactivation of enzyme was speculated due to conformational change as indicated by circular dichroism. Golovan et al., (2000) purified phytase from E. coli of 45 kDa molecular weight and further separated it into two isoforms of identical size with $\pi$ of 6.5 and 6.3 by chromatofocussing. The isoforms showed similar optimum temperature and pH of 60°C,
4.5 respectively. Greiner et al., (1993) purified two periplasmic phytases, P1 and P2, from *E. coli* to near homogeneity. The active protein was found to be monomer of 42 kDa. The hydrolysis pathway for P2 was deduced and identified as 6-phytase (EC 3.1.3.26) with pH optima of 4.5.

Phytases from *Klebsiella* sp. PG-2, *K. aerogenes* and *K. terrigena* were purified and characterized using ion exchange and gel filtration chromatography (Shah and Parekh, 1990; Tambe et al., 1994; Greiner et al., 1997). Tambe et al., (1994) isolated two isoforms of phytases from *K. aerogenes* that differed in molecular weight, pI, Km, thermostability, pH and temperature optima. It was reported to be the first instance where such a small, 10-13 kDa, fragment retained full phytase activity. Segueilha et al., (1992) purified extracellular phytase (490 kDa) from *Schwanniomyces castellii* using column chromatography. The native protein was reported to be tetrameric with one large subunit (125 kDa) and three identical small subunits (70 kDa) besides exhibiting glycosylation rate of 31%. The enzyme was optimally active at 77°C, pH 4.4 and was able to completely dephosphorylate phytate.

Generally, the phytases from bacteria have optimum pH in neutral to alkaline range while in fungi, optimum pH range is 2.5-6.0 and the stability of phytase decreased dramatically above 7.5 and below pH 3.0. This wide range of differences in pH optima could be due to variation in molecular conformation or stereo-specificity of the protein from different sources. Phytase shows high activity in the temperature range of 50-70°C while optimum temperature is mostly between 45-60°C.

### 4.1.1 Catalytic characterization

Phytases release free inositol and orthophosphate from phytic acid, besides various intermediary products *viz.*, the mono-, di-, tri-, tetra-, and penta-esters of inositol, thus, phytase activity can be determined either by measuring the release of orthophosphate or lower InsPs. Conventionally, inorganic phosphate liberated upon the action of phytase was measured by colorimetric method where phosphomolybdate formed was extracted out in organic solvent (Ullah, 1988). Recently, a reverse phase C18 HPLC methodology was developed for separation and quantitative determination of phytic acid and lower InsPs (Burbano et al., 1995). One unit of phytase activity was expressed as nanomoles of phosphorus released per ml per second *i.e.*, nkat/s (Ullah and Gibson, 1987). Phytases are
fairly specific for phytic acid under the assay condition and it is possible to distinguish phytase from acid phosphatase that is incapable of degrading phytate (Konietzny et al., 1995). The general enzymatic reaction as proposed by Holub (1982) is as follows:

Apart from inositol hexaphosphate, other polyphosphates formed during enzymatic reaction can be further hydrolyzed by phytase (Liu et al., 1998).

### 4.1.2 Kinetic and substrate selectivity

Kinetic parameters for dephosphorylation of phytate have been studied widely. The Michaelis constant \((K_m)\) for phytic acid varied from 17 \(\mu\)M for *Typha latifolia* (Hara et al., 1985) to 91 \(\mu\)M for maize (Feuillade and Dorizo, 1992) while *A. ficuum* phytase showed \(K_m\) of 40 \(\mu\)M (Ullah and Gibson, 1987). Though phytases are fairly specific for phytic acid, the substrate specificity may vary due to differences in molecular characteristics. Generally, the rate of enzymatic hydrolysis showed a classical Michaelis-Menten kinetics, \(i.e.,\) the liberaton of \(P_i\) is dependent on the substrate concentration used (Shimizu, 1992). It is generally recognized that inorganic phosphates cause product inhibition (competitive inhibition) of phytate hydrolysis (Konietzny et al., 1995; Greiner et al., 1993). Under certain circumstances, the substrate inhibition could be observed at phytate concentrations higher than 1.2 mM for *A. ficuum* (Ullah, 1988) and 0.3 mM for maize phytase (Hübel and Beck, 1996).

### 4.1.3 Detection of lower myo-inositolhexaphosphate isomers

The efficient analysis of inositol phosphates was found to be arduous, as these compounds do not absorb visible or ultraviolet light, nor can they easily be identified using specific colorimetric reagents. Conventionally, these compounds were determined by the method of Heubner and Stadler (1914) using ferric chloride as a precipitant. Unfortunately, the method was inadequate and lack the specificity needed for
distinguishing between the various isomers. After the development of ion-pair HPLC procedures, it became possible to study the hydrolytic products of inositolhexaphosphate (Sandberg and Ahderinne, 1986). These methods also could not differentiate isomeric forms of inositol phosphates, as gradient elution cannot be performed with RI detection. Mayr (1988) developed a novel complexometric dye- and transition-metal based post-column detection system for polyanions, called “metal-dye detection” that allowed isomer-selective determination of polyphosphorylated non-radioactive compounds in the picomolar range. At the same time, various InsPs, including all four pentakisphosphates and four tetrakisphosphates, were resolved by gradient ion chromatography coupled with postcolumn derivatization (Phillippy and Bland, 1988). The detection of myo-inositol and InsPs by HPLC using reverse phase C18 column with refractive index (RI) detector was reported (Sasaki et al., 1988; Lauro et al., 1989; Lehrfeld, J., 1989; Indyk and Woollard, 1994). A remaining problem, however, was to separate isomers from the whole spectrum of InsPs (IP1-IP6). Skoglund et al., (1997) developed a sensitive high performance ion chromatography (HPIC) method for the separation and quantitative determination of (IP1-IP6) isomers using high performance ion exchange columns with gradient elution and detection by either post-column reaction or chemically suppressed conductivity detection.

4.2 Sequence studies
The chemical sequencing of Aspergillus niger phytase (phy A) was reported by Ullah and Dischinger (1993a) while the phytase sequence of the cloned DNA from another strain, Aspergillus niger van teigham, was deduced and was found to be identical to phy A sequence (van Hartingsveldt et al., 1993). The mature Aspergillus ficuum phytase was found to possess ten potential N-glycosylation sites and consists of 441 amino acid residues that are composed of 37% nonpolar, 42% polar, 11.5% acidic and 9.5% basic amino acids (Figure 1). Ullah and Dischinger (1993a) suggested that tryptophan is essential for the phytase activity while disulphide bonds were crucial for maintaining the geometry of active site. Pasamontes et al., (1997) reported phytase from Emericella nidulans (463 amino acids; 51785 Da) and thermophilic fungus Talaromyces thermophilus (466 amino acids; 51450 Da). Both the proteins showed a high percentage of sequence identity to the phytases of A. niger (63 and 61%, respectively), A. terreus
9A1 (59 and 58 %), *M. thermophila* (50 and 48 %) and *A. fumigatus* (67 and 61 %). This allowed the modeling of fungal phytases leading to the identification of 21 amino acids conserved in fungal phy A. Tomschy *et al.*, (2000a) identified monomeric, glycosylated wild type phytase from *Aspergillus niger* T213 with a three-fold lower specific activity than *A. niger* NRRL 3135 phytase. Sequence comparison studies showed that two proteins differ at 12 amino acid residues, nine of which were distant from the active site and unlikely to influence the catalytic properties. Of the remaining three, the R297Q mutation was seen to account for the observed differences where R297 may result in stronger substrate and product binding and thereby, reduce the rate of product release during catalysis of T213 phytase. Thus, the sequence comparison studies and identification of candidate amino acids allowed an increase in specific activity of *A. fumigatus* phytase up to seven-fold (Tomschy *et al.*, 2000b).

4.3 Determination of active site

Phytases show remarkable homology with the active site residues of the members of acid-phosphatases *i.e.*, *Histidine Acid Phosphatases* (HAPs). Ullah *et al.*, (1991) characterized the residues involved in the active site formation of *A. niger* NRRL 3135. They studied the role of Arg in active center of phytase by carrying out inactivation studies using cyclohexanedione, a specific modifier of arginine and observed that the loss of phosphohydrolase activity was correlated with the loss of arginyl residues, suggesting that essential arginyl residues were involved in the catalytic site, rather than substrate-binding site. When *A. niger* NRRL 3135 N-terminal amino acid sequence was compared with other phosphatases, it was noted that a tripeptidic region (RHG) was present not only in fungal phy A but also in phy B (Ullah and Dischinger, 1993b). Ullah and Dischinger (1992) suggested the presence of histidine residues in the catalytic site of fungal phytase by chemical probing and also found that the oxidation of tryptophan residues by *N*-bromosuccinimide resulted in the inactivation of fungal phytase. There were four Trp residues in phy A (48, 290, 399, 462) and sequence comparison studies indicated the essential W290 residue in phy A that was preserved at position 290 in phy B (Ullah and Dischinger, 1995). When the sequences of phytases and acid-phosphatases from various sources *viz.*, phy A, phy B, *pho3*, *pho5* and pH 2.5 optimum acid
phosphatase in *E. coli*, lysosomal acid-phosphatase were compared, a conserved sequence of RHGXRXP was observed. Thus, proteins exhibiting this motif in the N-terminal site were grouped as “histidine acid phosphatases”. It was seen that *A. ficuum* NRRL 3135 phy A and phy B exhibit sequence similarities in the active site despite very low (19.5%) overall sequence homology (Ullah and Dischinger, 1993a; Ullah and Dischinger, 1993b). Further chemical probing of the active site indicated that both proteins react differentially to arginine modifiers that might be responsible for their different catalytic properties. This indicated that other parts of protein play a role in the active site formation, despite similar active site residues in these two proteins (Ullah and Sethumadhavan, 1998).

### 4.4 Phytase engineering studies

#### 4.4.1 Heat tolerance

The industrial importance of thermostable enzymes is increasing, thus, the isolation, characterization and engineering of enzyme besides the determinants of thermostability, are hotspots of current research. Thermostability is prerequisite for the successful application of enzymes in animal feed which are exposed to 60-90°C temperatures during pelleting process. Wyss *et al.*, (1998) analyzed the thermostability of three HAPs viz., *A. fumigatus*, *A. niger* phytase and *A. niger* pH 2.5 optimum acid phosphatase. During feed pelleting experiments, where 85°C temperature was reached, 51% of the recombinant *A. fumigatus* phytase activity was recovered as compared to 31% for *A. niger* phytase or 14% for pH 2.5 optimum acid phosphatase. The phytase genes from several thermophilic fungi have been cloned and expressed (Mitchell *et al.*, 1997; Pasamontes *et al.*, 1997; Berka *et al.*, 1998). Ullah and Mullaney (1996) reported that the inherent stability of *A. ficuum* phytase at 60°C was due to the presence of ten cysteine residues involved in the formation of five disulphide bonds. Thus, to further increase the thermostability, engineering of additional disulphide bridges may be a promising strategy.

Various compounds have been reported to enhance the stability of phytase at higher temperatures. Calcium was reported to contribute towards heat tolerance of phytases from many microbes (Kim *et al.*, 1998) while phytate enhanced the activity of *Arxula adeninivorans* phytase (Sano *et al.*, 1999). Chen *et al.*, (2001) demonstrated that 90% and
18% of phytase activity obtained at 37°C was retained at 70 and 80°C, respectively when sorghum liquor waste along with soluble starch (10:1) was used.

To improve the thermostability of phytase, immobilization of wheat phytase in polyacrylamide gel was carried out which stabilized the enzyme for 10 hour at 60°C, whereas the native enzyme lost 38% of its activity under similar conditions (Khare et al., 1994). The phytase from *E. coli* was covalently immobilized on NHS-activated Sepharose®, which enhanced the stability of enzyme against heat treatment (Greiner and Konietzny, 1996). Glycosylation was found to have significant impact on the stability of protein as reported by Han et al., (1999) for recombinant *A. niger* phy A expressed in *S. cerevisiae* and in *Pichia pastoris* (Han and Lei, 1999).

Recently, Lehmann et al., (2000a) used a novel consensus approach to increase the intrinsic thermostability of fungal phytases. Sequence comparison studies of 13 phytases from six different fungi and selection of most conserved position of each residue, led to the *de novo* construction of consensus phytase with drastically improved thermostability. The consensus phytase-1 had normal catalytic properties but showed an unexpected 15-22°C increase in unfolding temperature as compared to each of its parents. Structural comparison studies of consensus phytase-1 and *A. niger* phytase indicated that an unexpected direct link exist between protein sequence and protein stability. Later, they created a consensus phytase-7 by replacing all the divergent active site residues of consensus-1 phytase with the respective residues of *A. niger* NRRL 3135 phytase. Although decrease in unfolding temperature was observed but consensus phytase-7 was still 7°C more thermostable than various wild type phytases known till to date. Thus, combination of this approach along with selection of “preferred” active site allows the design of thermostable variant possessing favorable catalytic properties of HAPs (Lehmann et al., 2000b)

### 4.4.2 Temperature and pH optima

The fungal phytases, generally, have temperature optima between 45-55°C being 58°C for *A. niger* NRRL 3135 phytase. Lehmann et al., (2000a) carried out the *de novo* construction of consensus phytase-1, using novel consensus approach and the purified enzyme was found to have temperature optimum of 71°C, thus an increase of 16-26°C was obtained than that of various parent phytases. Using differential scanning
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calorimetry, an unfolding temperature ($T_u$) of 78°C was measured for consensus phytase, which was 15-22°C higher than $T_u$ measured for various parent phytases. The pH activity profile of consensus phytase (optimum at pH 6.5) differed most from those of *A. terreus* and *Myceliophthora thermophila* and was most similar to that of *E. nidulans* phytase (Mitchell *et al.*, 1997; Wyss *et al.*, 1999b). Later, they constructed consensus phytase-7 and studied the effect of assay buffers on the phytase activity. *A. niger* NRRL3135 phytase is the only phytase having dual pH optima (2.5 and 5.0-5.5). In all these studies, acetate, borax-succinate buffers in the pH range 3.5-4.5 were used but when citrate was used as buffer, the local activity minimum at pH 4.0 was no longer observed, instead, the two peaks fused to give a single pH optimum (Lehmann *et al.*, 2000b). Thus, engineering phytases with broad pH optimum for phytase activity, as reported for *A. fumigatus* phytase (Pasamontes *et al.*, 1997a), would expand the enzyme’s usefulness as feed additive where it should be able to counteract the pH level found in the digestive tract of animal besides retaining activity at lower body temperatures.

4.4.3 Enzyme stability

Wyss *et al.*, (1999a) cloned and over expressed wild type phytases from six different fungi in yeast and filamentous fungi and found that several fungal phytases, when expressed in *A. niger*, were susceptible to limited proteolysis which invariably occurred at the exposed loops on the surface of the molecule. They successfully engineered higher level of proteinase resistance found in *A. niger* phytase into *A. fumigatus* and *E. nidulans* phytase by site-directed mutagenesis at those cleavage sites. Therefore, engineering of surface-exposed loops may be a promising strategy for improving phytase stability during feed processing against proteolytic attack.

Glycosylation is one of the major naturally occurring modifications in the covalent structure of proteins resulting in the stabilization of protein confirmation, protection from proteolysis and enhancement of nascent polypeptide solubility. It was seen that generally, deglycosylation decreases protein stability and maximum effect was observed in case of heavily glycosylated proteins, irrespective of the types (N-linked or O-linked) or patterns (mono or multi-branched) of covalently attached carbohydrate chains (Wang *et al.*, 1996). Han *et al.*, (1999) studied the expression of *A. niger* phytase (phy A) in *S. cerevisiae* and the effect of glycosylation on phytase activity and stability. The expressed
phytase was heavily glycosylated, deglycosylation of which resulted in the loss of 9% phytase activity and 40% stability at higher temperatures. Lehmann et al., (2000a) used a novel consensus approach for the de novo construction of consensus phytase-1 that had improved intrinsic catalytic properties and determined its crystal structure at 2.9 Å resolution. Stabilization of surface loop having consensus amino acid sequence of T249(S), S250(T), D251(V), A252(D), T253(T) was observed in consensus phytase due to intramolecular interactions. Replacement of valine by aspartic acid was found to be most important single mutation, resulting in the stabilization of loop by hydrogen bonding, besides contributing towards stabilization of the N-terminus.

4.4.4 Synergistic effect

It was observed that when Aspergillus niger NRRL 3135 was grown under phosphate limiting conditions, three extracellular phytases were produced namely phy A (Ullah and Gibson, 1987), phy B (Ullah and Cummins, 1987), pH 6.0 acid phosphatase (Ullah and Cummins, 1988; Mullaney et al., 1995). Their simultaneous expression in A. niger indicated that the organism utilizes all these enzymes to scavenge phosphorus from the environment under phosphate starvation conditions, when a single phosphatase/phytase will not be sufficient enough to meet the requirements. Moore et al., (1995) cloned and expressed A. niger aphA gene in A. oryzae, which resulted in five-fold higher phytase activity than the control wild type A. oryzae strain, indicating that the increase was due to synergistic effect between recombinant purple acid phosphatase and the host A. oryzae phytase. Mullaney and Ullah (1998) suggested that mixture of Apase6 and phytase might enhance their effectiveness as animal feed additives to reduce phosphate pollution due to livestock units. Park et al., (1999) reported that combination of A. niger NRRL 3135 phy A and B. amyloliquefaciens phy C would be much more effective in hydrolyzing phytate than either single phytase alone. Wyss et al., (1999b) also showed that phy A was able to release five phosphate groups from phytic acid but the combination of phy A and phy B was able to release all the six phosphate groups. Meittinen-Oinonen et al., (1997) over expressed A. niger phy B in Trichoderma reesei, a high- cellulase producing strain under the control of cellobiohydrolase producing (cbh I) promoter which increased the secretion 240 times more than wild type strain. When the recombinant enzyme was employed in feed-fodder having significant amounts of phytic
acid and cellulose, it resulted in their improved bioavailability. Similarly, Zyla et al., (1995) studied the enzymic "cocktail" comprising phytase, acid-phosphatase, acid-protease, pectinase and citric acid, under simulated intestinal conditions to dephosphorylate corn and found improved digestibility of phytate phosphorus, proteins and carbohydrates. Thus, the combination of enzymes would be beneficial in increasing phosphorus utilization in animal feeds having high phytate content.

4.5 Immobilization studies
Phytases act sequentially on myo-inositolhexakisphosphate to liberate various lower isomers of InsPs (InsP₅, InsP₄, InsP₃, InsP₂, InsP). Thus, an efficient bioreactor of immobilized phytase could be used to produce various isomers of phytic acid besides rendering the molecule non-chelator of metal-ions, proteins etc. Ullah and Cummins (1987) constructed a packed-bed bioreactor using A. niger NRRL 3135 phyA, covalently immobilized on Fractogel TSK HW-75F. Though no shift in pH optima was observed but temperature optima shifted from 58 to 65°C with an increase of Kₘ for phytate and 50% more release of phosphorus from phytate. When the products were analyzed by HPLC, only InsP₂ and InsP were detected in the eluate after repeated hydrolysis of phytate by the bioreactor (Ullah and Phillippy, 1988). Immobilization of extracellular A. ficuum phytase on glutaraldehyde-activated silicate resin resulted in the improved performance of phytase at higher temperatures as compared to soluble enzyme, exhibiting maximum catalytic activity at 65°C than 58°C for soluble enzyme (Ullah and Cummins, 1988). In an attempt to immobilize phytase through its carbohydrate moieties rather than protein backbone, it was observed that the diminished phytase activity, bioreactor’s output and the Kₗ resulted when the attachment was made through protein backbone due to the distortion of active center by extensive cross-linking of proteins to the matrix and as the native phytase is heavily glycosylated, prevention of extensive cross-linking is difficult. Thus, by site-directed mutagenesis, it should be possible to remove glycosyl residues to produce an enzyme that can be immobilized by a few carbohydrate moieties while retaining a high level of phytase activity (Dischinger and Ullah, 1992). Greiner and Konietzny (1996) covalently immobilized E. coli phytase on NHS-activated Sepharose® which resulted in the improved heat tolerance at 70 and 80°C. Even after 1 hour, no loss
of enzyme activity was observed while free phytase showed no remaining activity at 70 and 80°C, the immobilized phytase retained 42% of remaining activity. Liu et al., (1999) increased the temperature optima of A. ficuum phytase to 58°C, which was 8°C higher than that of free enzyme, by immobilization in gelatin gels and further hardening with formaldehyde. Apparent $K_m$ increased to 3.28 mM from 2.34 mM for free enzyme while only 34.6% of the free enzyme activity was retained after immobilization.

4.6 Structural studies
The crystal structure of E. coli phytase was determined by two-wavelength anomalous diffraction method using strong tungsten scattering. Despite a lack of sequence similarity, the structure closely resembles the overall fold of HAPs (Lim et al., 2000). Jia et al., (1998) carried out crystallographic studies of recombinant E. coli phytase, a 6-phytase, to reveal the underlying structural basis for the different catalytic properties between 3- and 6-phytase. Crystals were obtained by bulk-crystallisation method without using conventional precipitants and diffracted at 2.2 Å X-ray source while data set was collected to 2.3 Å resolution. Ha et al., (1999) carried out X-ray crystallographic analysis of Bacillus amyloliquefaciens phytase, which showed no homology to other known phytases and phosphatases, using hanging-drop vapor-diffusion method. As the enzyme showed requirement of Ca$^{2+}$ for the catalytic activity and thermostability, therefore, to further understand the role of Ca$^{2+}$ the structure of novel, thermostable phytase in both the partially and fully Ca$^{2+}$ loaded states at 2.1Å resolution was determined. They proposed a new folding architecture for phosphatase activity where the presence of two Ca$^{2+}$ at high affinity binding sites resulted in dramatic increase in thermal stability by as much as 30°C while binding of three additional Ca$^{2+}$ to low affinity binding sites at the top of the molecule, turns on the catalytic activity of the enzyme by converting the highly negatively charged cleft into a favorable environment for the binding of phytate (Ha et al., 2000). Aspergillus ficuum (phy A) phytase is a very important member of HAPs and was widely investigated by several authors. As phy A and phy B were reported to contain 10 and 8 cysteine residues, respectively (Ullah and Dischinger, 1993; Ehrlich et al., 1993), Ullah and Mullaney (1996) examined the role of disulphide bonds in the formation of catalytic active center in phy A by studying the unfolding-refolding of enzyme at
different pH levels in the presence and absence of β-mercaptoethanol. It was found that disulphide bonds, indeed, play crucial role in maintaining the geometry of active center and any perturbation of their formation result in catalytic demise. Kostrewa et al., (1997) reported crystal structure of phytase from \textit{Aspergillus ficuum} at 2.5 Å resolution which consisted of 7-248 and 253-444 amino acid residues, 115 water molecules and one sulphate binding site distant from the active site. All the ten-cysteine residues were involved in five disulphide bridges that played important role in maintaining the structural integrity of active site. The structure was divided into two domains: larger α/β and smaller α domain. The α-domain was found to have a new fold, distinctly different from other phosphatases.

5. CLONING AND EXPRESSION OF PHYTASE

The N-terminal and intersequences of \textit{A. ficuum} phytase (Ullah, 1988) led to the cloning of phy A gene from \textit{A. niger} NRRL3135 in λgt11 expression vector (Mullaney et al., 1991). The full-length gene was subsequently cloned and the sequence was submitted to GenBank (Acc. No. M94550). At the same time, van Hartingsveldt et al., (1993) cloned and expressed phy A gene from \textit{A. niger} NRRL3135 using degenerate oligos based on phytase amino acid sequence. It was seen that after the culture growth under phosphate starvation conditions, transcription of 1.8 kb fragment was initiated and expression of multiple copies of phy A gave up to 10-fold higher phytase activities than native wild type strain. However, the cloning of \textit{A. niger} phy A gene in \textit{E. coli} resulted in inactive, non-glycosylated phytase (Phillippy and Mullaney, 1997). Later, Han et al., (1999) studied the expression of \textit{A. niger} phy A in \textit{S. cerevisiae} by inserting 1.4 kb DNA fragment and found that the yield of extracellular phytase was affected by the signal peptide and the medium composition. The expressed phytase had dual pH optima of 2.0-2.5 and 5.0-5.5 with temperature optimum between 55-60°C for catalytic activity. Due to heavy glycosylation, the expressed phytase had molecular weight of 120 kDa, deglycosylation of which resulted in the loss of 9% phytase activity and 40% thermostability. At the same time, they studied the expression of phy A in \textit{Pichia pastoris} by inserting 1.4 kb fragment and found that high level of active phytase (25-65 μmol/min/ml) secreted into the medium. The suppression of glycosylation by
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tunicamycin resulted in significant reduction of phytase activity, thus suggesting that, indeed, glycosylation was vital to the biosynthesis of phy A (Han and Lei, 1999).

Ehrlich et al., (1993) cloned second phytase gene (phy B) from A. niger NRRL 3135 by probing DNA λEMBL3 library with 354-bp DNA fragment based on partial amino acid sequence of a pH 2.5 optimum acid phosphatase. A clone containing 1.6 kb fragment (phy B) encoding 479 residue protein with a leader peptide of 19 amino acids was isolated. The sequence comparison studies showed 23.5% homology with phy A besides sharing high degree of homology at amino acid 81, containing “RHGXRXP” motif and at 336 containing “HD” dipeptide. Later, an acid-phosphatase that could not be readily separable from pH 2.5 optimum acid phosphatase (phy B) was cloned and found to be homologous to Penicillium chrysogenum phoA (Ehrlich et al., 1994). Piddington et al., (1993) cloned and sequenced phytase and pH 2.5 acid-phosphatase from A. niger var awamori. A 102 bp intron was identified between the start codon and the N-terminus besides a 19-residue signal peptide in the N-terminal segment. A clone containing 1.8 kb fragment was sub cloned and nucleotide sequence revealed the regions coding for amino acid sequences of known phytase peptides. Another phytase with undefined active site, showing no homology to known HAPs was identified, characterized and cloned from Bacillus subtilis VTT E-68013 using degenerate primers. Sequences of positive clones carrying 6 and 2.4 kb inserts were determined using vector-specific and gene-specific primers. PhyC did not have the conserved RHGXRXP motif found in the active site of HAPs and thus, appeared to be novel phytase (Kerovuo et al., 1998).

Pasamontes et al., (1997a) cloned, purified and characterized heat-stable phytase from a thermophilic fungus, A. fumigatus. An insert of 1.571 kb carrying the complete phy A was isolated and transformed into A. niger which encoded phytase (60 kDa) of 465 amino acids with first 26 amino acids representing signal sequence. The purified enzyme showed high activity with phytic acid in the pH range of 2.5-7.5 and was able to withstand temperatures up to 100°C over a period of 20 minutes, with a loss of only 10% of the initial enzymatic activity. Later, they cloned phytases from thermophilic fungi, Emericella nidulans and Talaromyces thermophilus. Both the proteins exhibited high sequence homology (48-67%) to known phytases (Pasamontes et al., 1997b).
Berka et al., (1998) cloned phy A gene from thermophilic fungus, *Thermomyces lanuginosus*, by inserting it into the expression vector under *Fusarium oxysporum* trypsin gene promoter and heterologously expressed in *Fusarium venenatum*. The phy A gene encoded 475 amino acids peptide, which included a putative signal peptide (23 amino acid) and propeptide (10 amino acid). The secreted recombinant phytase was enzymatically active between pH 3.0-7.5 and showed superior catalytic efficiency to any of known fungal phytase at 65°C (optimum temperature).

Golovan et al., (2000) cloned phytase gene from *E. coli* ATCC 33965 and showed that the acid-phosphatase *appA* gene encode bifunctional enzyme exhibiting both phytase and acid-phosphatase activity. The enzyme was first reported and purified by Dassa et al., (1982), exhibiting optimum pH of 2.5 for catalytic activity. Later, cloning of *appA* gene encoding *E. coli* APase was carried out (Dassa and Boquet, 1985) and the complete nucleotide sequence of the *appA* gene (GenBank Acc. No. M58078) was determined which revealed significant homology between pH 2.5 acid-phosphatase and glucose-1-phosphatase (Dassa et al., 1990). Greiner et al., (1993) purified and characterized phytase from *E. coli* ATCC 33965 and reported that the kinetic properties of native purified phytase were similar to those of *appA* encoded acid phosphatase. Golovan et al., (2000) cloned phytase gene by PCR using primers based on published *appA* sequence of *E. coli* strain K12 (GenBank Acc. No. M58078). Screening of genomic library revealed three distinct clones: one contained *appA*, having high phytase activity; second insert contained *agp*, encoding periplasmic acid glucose-1-phosphatase that showed significant homology to *appA* phytase; and the third clone exhibited phytase activity when the insert was expressed in *E. coli* DH5α while no activity was detected when introduced in *appA*-negative strain of *E. coli*. The isoforms were found to possess identical pH and temperature optimum for catalytic activity (pH 4.5 and 60°C) with molecular mass of 44,708 Da but slightly varied in their isoelectric points (6.3 and 6.5).

Tye et al., (2002) cloned a novel phytase gene (*phyL*) consisting of 1,146 bp coding region with an upstream 300 bp promoter region from *Bacillus licheniformis* by multiple steps of degenerate and inverse PCR. The phytase encoded by *phyL* exhibited higher thermostability, retaining 80% of its original activity after denaturation at 95°C, thus, suggesting it to be suitable candidate for animal feed applications and transgenic studies.
6. APPLICATION OF PHYTASES

6.1 Phytase as feed additive

Since phytic acid cannot be metabolized by monogastric animals due to lack of phytate degrading enzyme in their gastrointestinal tract, therefore, feeds for pigs and poultry are commonly supplemented with inorganic phosphate in order to meet the phosphorus requirements (Reddy et al., 1982). It was seen that use of phytase in feed-fodder improves the phosphorus bioavailability besides resulting in the loss of chelating ability of phytic acid and reducing phosphorus excretion in the areas of intensive livestock (Yano et al., 1999). The superior activity of *Aspergillus niger* NRRL 3135 phytase and its practical application to animal feed for the removal of phytic acid was demonstrated in several studies (Howson and Davis, 1983; Han, 1988; Han and Wilfred, 1988). Simons et al., (1990) and Jongbloed et al., (1992) collaborated to confirm the earlier feed studies by feeding solvent precipitated phytase to chick diets besides demonstrating the efficacy of feeding phytase to swine. They concluded that the addition of enzyme (1000 μmole P/h/ml phytase /kg diet) was sufficient to improve performance than that obtained by adding supplemental inorganic phosphate, thus establishing the role of phytase as feed-additive in combating phosphorus pollution in soil and water.

The Natuphos produced by Gist-Brocades, when supplemented in feed, resulted in enhanced utilization of phytin phosphorus by monogastric animals (Broz et al., 1994). Alko Biotech tested FinaseF for its use in improving the phosphorus availability in swine and found parallel results as those obtained by phytases from other sources (Young et al., 1993; Lei et al., 1993 & 1994). It was observed that the units of enzyme required for the hydrolysis of set amount of phytin phosphorus were fairly consistent and can estimate within a narrow range, the amount of phytase required to hydrolyze phytin-P present in any commercial diet used in livestock units. Depending upon the specific diet, 380-1000 μmole P/h (~ 6308-16600 nkat/ml) phytase is required to replace 1 gram of phosphorus supplied by inorganic source. It was seen from the livestock production statistics, USDA that if phytase were used as feed ingredient in the diets of all the monogastric animals in United States, it would release phosphorus that has a value of $ 1.68 \times 10^8$ per year besides diminishing the amount of phosphate in the manure and subsequently entering the environment. The quantitative determination of released phosphate upon phytase
supplementation indicated that if the phytase were used in the diets of all monogastric animals in U.S., it would preclude $8.23 \times 10^7$ kg phosphorus from entering the environment (Wodzinski and Ullah, 1996).

6.2 Preparation of myo-inositol phosphates

Besides the adverse effects of phytate and other highly phosphorylated inositol phosphates on mineral bioavailability, some novel metabolic effects of some of its degradation products have been recognized. The most extensively studied positive aspect of myo-inositol phosphate ($\text{InsP}_1, 4, 5$ and $\text{InsP}_1, 3, 4, 5$) is their potential for reducing the risk of colon cancer. $\text{InsP}_5$ play important role in modulation of the oxygen binding affinity of hemoglobin in the erythrocytes of avian, amphibian and reptilian species (Bartlett, 1982) while $\text{InsP}_4$ along with $\text{InsP}_3$ was found to act as secondary messenger in synergistically controlling intracellular calcium level (Berridge and Irvine, 1989). The position of phosphate group on inositol ring is, thereby, of great significance for their physiological function. To investigate the physiological effects of defined myo-inositol phosphate isomers, these compounds have to be available in pure form and sufficient quantity. Attempts to produce them non-enzymatically results in the mixture of isomers, thereby making the purification very uneconomical and arduous task. Thus, a better way to produce these isomers would be to use phytases that are capable of sequentially hydrolyzing phytate. As phytases are distributed widely in nature and different type of phytases are known, 3-phytases, 4-phytases and 6-phytases, indicating the predominant attack of susceptible phospho-ester bond. Thus, using phytases of different origin may lead to production of different isomers. Siren (1986) prepared myo-inositol-1,2,6-triphosphate and myo-inositol-1,2,5-triphosphate by enzymatic hydrolysis of phytic acid using $\text{S. cerevisiae}$ phytase. The esters of inositol triphosphate were found to alleviate conditions associated with abnormal levels of NeuropeptideY (NPY) such as arthritis and asthma besides acting as a pain killer (Siren et al., 1992; Siren, 1995).

Greiner and Konietzny (1996) reported the synthesis of $\text{Ins-1,2,3,4,5-P}$, $\text{Ins-2,3,4,5-P}$, $\text{Ins-2,4,5-P}$ and $\text{Ins-2,5-P}$ using $\text{E. coli}$ phytase covalently immobilized on NHS-activated Sepharose®. As only one major isomer of each InsP was formed, further purification could be easily achieved by ion-exchange chromatography. Using a
combination of HPIC and kinetic studies, the stereo specificity of \textit{E. coli} phytase P2 was established indicating the sequential removal of phosphate groups via 6/1/3/4/5 route (Greiner \textit{et al.}, 2000).

6.3 Pulp and Paper industry
It has been speculated that the removal of plant phytic acid might be important in the paper and pulp industry. A thermostable phytase could have the potential as a novel biological agent to degrade phytic acid during pulp and paper processing. The enzymatic degradation of phytic acid would not result in the production of toxic and mutagenic by-products. Therefore, exploitation of phytases in the paper and pulp process could be eco-friendly and would assist in the development of cleaner technologies (Liu \textit{et al.}, 1998).

6.4 Phytase as soil amendment
The phytic acid and its derivatives represent up to 50\% of the total organic phosphorus in the soil at certain locations, suggesting the possibility of phytase addition that might stimulate plant growth in these soils. Findenegg and Nelemans (1993) studied the effect of phytase (phy A) on the availability of phosphorus from phytic acid in the soil for maize plants. It was seen that phytin hydrolysis increased when phytase was added to the soil which further resulted in stimulation of plant growth. However, the amount of phytase necessary for a significant effect indicated that it is not a practical technique. Thus, expression of phytase in the roots of plants might increase the phosphorus bioavailability (Day, 1996).

6.5 Semisynthesis of peroxidase
Based on structural similarity between the active site of vanadium-dependent haloperoxidase, fungal phytase and acid-phosphatase, a semi synthetic peroxidase was designed (van de Velde \textit{et al.}, 2000). The Delft group incorporated vanadate ion into the active site of \textit{A. niger} NRRL 3135 phytase, thus transforming native phosphohydrolase into semisynthetic peroxidase. The “new” enzyme was able to catalyze enantioselective oxidation of prochiral sulphides and was found to be stable for 3 days with only slight loss in turnover number. Being water miscible, the enzyme could be used in the presence
of organic solvents up to 30% concentration (v/v) with only slight loss in enzyme activity. It was seen that of all the phytases and acid-phosphatases tested, only HAPs with “RHGXRXP” active site sequence could function as peroxidase when vanadate ion was incorporated into the active site.

7. RESEARCH ON PHYTASE AND ITS COMMERCIALISATION

7.1 In world

The research on phytase started when Suzuki et al., (1907) extracted phytase from rice bran and Dox and Golden (1911) demonstrated that Aspergillus sp. also produce phytase. It was recognized that phytin was an effective chelating agent and antinutrient for monogastric animals. Thus, attempts to hydrolyze phytin in animal feed using live yeast were made but the first concrete effort to make phytase a commercial product started in 1962 at International Minerals and Chemicals, Skokie. Sheih and Ware (1968) isolated Aspergillus ficuum that produced highest yield of phytase and deposited as NRRL 3135. Shieh et al., (1969) developed the strain, A. niger NRRL 3135, for production of phytase that had two pH optima, 5.5 and 2.5. The microbial biochemistry group at IMC scaled the process to 114 L stage and animal nutrition group tested the enzyme for its use as feed additive. Unfortunately, at that time, the yields of phytase were not high enough to produce a product that would be competitive with the feeding of inorganic phosphorus hence the project was terminated in 1968.

7.1.1 USDA studies

In 1980s, methodology for cloning of genes and the use of efficient promoters in gene constructs to increase the phytase yield was developed. In 1984, the technology developed at IMC was transferred to United States Department of Agriculture (USDA), Agricultural Research Service (ARS), Southern Regional Research Center, (SRRC) and the research team at SRRC, in 16 years of time span, isolated, characterized and sequenced phytases (phy A and phy B) and acid-phosphatases produced by A. niger NRRL 3135. The N-terminal and intersequences of A. ficuum phytase (Ullah, 1988) led to the cloning of phy A gene from A. niger NRRL3135 in λgt11 expression vector (Mullaney et al., 1991). The full-length gene was subsequently cloned and deposited to Genbank (Acc.No. M94550). At the same time, van Hartingsveldt et al., (1993) cloned
and expressed phy A gene from *A. niger* NRRL 3135 and the expression of multiple copies of phy A gave up to 10-fold higher phytase activities than native wild type strain. A second phytase (phy B) was cloned and expressed by Ehrlich *et al.*, (1993) and the encoded amino acid sequence was verified by the chemically deduced protein sequence (Ullah *et al.*, 1994).

### 7.1.2 Dutch studies

The Dutch group initiated the cloning of phytase in late 1980s after the biochemical and protein sequence information was published by the USDA group (Ullah, 1988). The researchers at Gist-Brocades cloned, sequenced and over-expressed phy A from *A. niger* NRRL 3135 along with amyloglucosidase promoter which resulted in 52-fold improvement of phytase yield. They also cloned the enzyme along with amyloglucosidase promoter and *A. niger* NRRL 3135 leader sequence into *A. niger* CBS 513.88, which resulted in 1400-fold improvement of phytase yield in one of the wild type non-producers (vanGorcom *et al.*, 1991). This bioengineered strain secreted 7.9 g/L of purified phytase with a specific activity of 2100 nkat/mg protein. It was seen that the cloned gene was identical to the one cloned by USDA group (GenBank Acc. No. M94550). The nutrition group at Gist-Brocades tested the bioengineered enzyme extensively in swine and poultry. After receiving approval from several countries and FDA as GRAS (Generally Recognized As Safe) for use in food, phytase is being marketed as food additive in the United States from January 1996 as Natuphos®.

### 7.1.3 Pan lab studies

The researchers of Alko Ltd., Finland started work on phytase at approximately same time for use in baking process and animal feeds. They enlisted the group at Pan Labs for cloning the second phytase gene (phy B) and increased the yield by cloning procedures. These groups cloned the genes for phytase and pH 2.5 optimum acid-phosphatase from *A. niger* var awamori (Piddington *et al.*, 1993). The genes were isolated from genomic DNA using probes based on protein sequences published by the USDA group and found that the gene encoding *aph* was similar to one coding for second phytase (phy B) in *A. niger* NRRL 3135 (Ehrlich *et al.*, 1993). Meittinen-Oinonen *et al.*, (1993) over-expressed pH 2.5 acid-phosphatase in *Trichoderma reesei* expression system and the enzyme is now available in market as Finase-F.
7.1.4 Novo-Nordisk studies

Lassen et al., (1997) reported phytase from a basidomycete, *Peniophora lycii*, and expressed in *Aspergillus oryzae* IFO 4177 (WO9828409 and US6060298). Based on protein sequence comparison, it was found to be similar to *A. niger* NRRL 3135 phy A with 10 cysteine residues forming disulphide bridges. Only the two N-terminal cysteine residues were found to be different in *P. lycii* phytase, besides, being recognized as 6-phytase unlike *A. niger* phytase which is a 3-phytase. The initial studies suggested its capability of releasing phosphate from phytic acid at high initial rate coupled with high specific activity. The enzyme is awaiting approval for marketing as Bio-Feed® phytase by Novo-Nordisk.

7.2 Current status in India

In India, Powar and Jagannathan (1982) first reported the production and purification of phytate specific phosphatase from *Bacillus subtilis*. India is presently using dicalcium phosphate (DCP) in animal feeds and it has been seen that phytase supplementation can replace 50-60% dicalcium phosphate. It is estimated that 10 kg DCP can be replaced by 250 gm phytase enzyme, thus, knowing the fact that 50-60% DCP can be replaced by phytase, the potential demand for phytase in cattle and poultry feed will be around 4000 tonnes per annum. However, as not all the livestock units depend upon commercial feed, therefore, the demand level is approximately 200 tonnes per annum (www.tidco.com).

7.2.1 Supply scenario

Indian requirement of phytase enzyme is largely imported and supplied by Novo-Nordisk which now tied up with Ranbaxy for marketing their product “BioFeed®” in India. Several Indian companies claim to produce phytase like Biocon India Ltd., Bangalore; Maps India Ltd., Ahmedabad and Textran Chemicals, Chennai. According to Tamil Nadu Industrial Development Corporation Ltd., the quality of phytase produced, in terms of product purity, by Indian units is not equivalent to that of Novo-Nordisk. The phytase produced by Indian units contain other enzymes such as cellulase and others, and it was observed by several authors that it would be advantageous to contain other hydrolytic activities besides phytase such as endo-glucanase, to assist in the digestion of the high molecular weight sugar polymers present in the grains. Meittinen-Oinonen *et al.*, (1997)
over expressed *A. niger* phy B in a heterologous host, *Trichoderma reesei*, which when employed in feed-fodder having significant amounts of phytic acid and cellulose, resulted in their improved bioavailability. Similarly, Zyla et al., (1995) studied the enzymic “cocktail” comprising phytase, acid-phosphatase, acid-protease, pectinase and found improved digestibility of phytate phosphorus, proteins and carbohydrates.

### 7.2.2 Present demand level

India is having demand of 200 tonnes of phytase and due to lack of indigenous technology for the phytase production, there is an ongoing research with the ultimate aim to produce phytase at cost-effective level. During the field survey, it was evident that DCP content in the cattle feed is 2% and considering that 5% of potential demand may exist, the potential demand for DCP by feed industry was evaluated to be $1.4 \times 10^5$ tonnes per annum (cattle feed) and $1 \times 10^5$ tonnes per annum (poultry feed). It was seen that the cost of BioFeed® in U.S. is $1.36$ per pound, which comes down to Rs 250 per kg. Textran Chemicals, Chennai sell their phytase at Rs 175 per kg that might be unaffordable to many livestock units (www.tidco.com). Thus, isolation of hyper producing organisms and expression of phytase genes to improve phytase productivity is still an ongoing research area.